Eur. J. Biochem. *145*, 365–371 (1984)

Glucose-6-phosphate dehydrogenase

A transferred nuclear Overhauser enhancement study of $NADP^+$ conformations in enzyme-coenzyme binary complexes

Angela M. GRONENBORN, G. Marius CLORE, Lynda HOBBS, and Jonathan JEFFERY

Division of Physical Biochemistry, National Institute for Medical Research, London; and Department of Biochemistry, University of Aberdeen

(Received July 13, 1984) – EJB 84 0769

The conformation of $NADP^+$ in glucose-6-phosphate-dehydrogenase–$NADP^+$ binary complexes has been investigated using proton-proton transferred nuclear Overhauser enhancement measurements to determine inter-proton distance ratios between bound $NADP^+$ protons. The enzymes from *Saccharomyces cerevisiae* (brewer's yeast and baker's yeast) and *Hansenula jadinii* (*Candida utilis*, *Torula utilis*) form binary complexes with $NADP^+$ in which the glycosidic bond of the adenine moiety is in the *anti* conformation whereas that of the nicotinamide moiety exists as a *syn* (69–70%)/*anti* (30–40%) mixture. The enzymes have similar subunit sizes ($M_r \approx 58000$) and it is shown that they bind $NADP^+$ in essentially similar conformations. Inactivation of the baker's yeast enzyme with acetylsalicylic acid caused little if any alteration in the conformation of bound $NADP^+$, and the presence of $NADP^+$ during inactivation afforded very little protection to the enzyme. Inactivation rates were, however, lower in the presence of glucose 6-phosphate. It is concluded that the ε-amino group of the lysine residue that is acetylated during the inactivation reaction with acetylsalicylic acid is not necessary for binary complex formation between the enzyme and $NADP^+$, but that it is situated in a part of the molecule affected by formation of the enzyme–glucose-6-phosphate complex. The implication of the findings for the catalytic process, and related evolutionary aspects, are discussed briefly.

Glucose-6-phosphate dehydrogenase has more known genetic variants than any other mammalian enzyme [1]. Their distribution in human populations has been influenced by association of low-activity forms of the enzyme with erythrocyte conditions unfavourable for the malaria parasite (*Plasmodium falciparum*) [2], and many people throughout the world have low-activity variants which put them at risk of haemolytic crises following treatment with certain drugs [3], or merely eating the seeds of the common vegetable broad beans (*Vicia faba*) [4].

A primary structure has been given (so far without experimental evidence) for most of the 495 residues of the normal human erythrocyte enzyme [5], though the small part of the structure for which there is published evidence is not agreed upon [6, 7]. Recently, the glucose-6-phosphate dehydrogenase of baker's yeast (*Saccharomyces cerevisiae*) was shown to be inactivated by acetylsalicylic acid, with transfer of the acetyl group to the ε-amino group of a lysine residue [8]. An eight-residue sequence containing this 'essential lysine' was identical with residues 93–100 of the sequence given by Beutler [5] for the human erythrocyte enzyme, strongly suggesting that these proteins are related [8]. The glucose-6-phosphate dehydrogenase of *Leuconostoc mesenteroides*, however, has uncertain and possibly different relationship [8, 9]; enzymes that catalyse the same reactions are not always closely similar proteins (cf. the different glutamate dehydrogenases [10], alcohol dehydrogenases [11], and polyol dehydrogenases [11]).

In the case of the glucose-6-phosphate dehydrogenases, estimates of subunit M_r have ranged from 10000 for a *Hansenula jadinii* enzyme [12] to 67000 for the pig liver enzyme [13]. Even for the enzyme from human erythrocytes, estimates range from 43000 [14] to 59000 [15], while values for the *S. cerevisiae* enzyme are from 22000 [16] up to about 58000 [8].

Investigation of glucose-6-phosphate dehydrogenases from several sources is therefore necessary, and studies on the enzymes from three yeasts are now reported. Two of these organisms (baker's yeast and brewer's yeast) are closely related (both *S. cerevisiae*) whereas the third (*H. jadinii*) is a different kind of yeast [17]. All three are organisms of industrial importance. Use of the transferred nuclear Overhauser enhancement (TRNOE) [18, 19] to investigate the conformation of the bound coenzyme $NADP^+$ is described for these three active enzyme, and, in the case of baker's yeast, also for enzyme inactivated by acetylsalicylic acid.

Abbreviations. NOE, nuclear Overhauser enhancement; TRNOE, transferred nuclear Overhauser enhancement.

Enzymes. Alcohol dehydrogenases (EC 1.1.1.1); dihydrofolate reductase (EC 1.5.1.3); glucose-6-phosphate dehydrogenase (EC 1.1.1.49); glutamate dehydrogenase (EC 1.4.1.2); lactate dehydrogenase (EC 1.1.1.27); polyol dehydrogenases: iditol and sorbitol dehydrogenases (EC 1.1.1.14) and ribitol dehydrogenase (EC 1.1.1.56).

MATERIALS AND METHODS

Materials

Brewer's yeast glucose-6-phosphate dehydrogenase was obtained from Pharmacia P-L Biochemicals (Piscataway, NJ,

USA), the baker's yeast and *Hansenula jadinii* enzymes from Sigma (St Louis, MO, USA). (*Hansenula jadinii* is the systematic name for a species of yeast also described as *Candida utilis* and *Torula utilis* [17].) Brewer's yeast and baker's yeast are varieties of the species *Saccharomyces cerevisiae* [17].

Glucose 6-phosphate, $NADP^+$, and Tris were from Sigma or Boehringer (Mannheim, FRG); deuterium oxide was from Norsk Hydro (Norway). Other chemicals and solvents were appropriately pure grades from Aldrich (Gillingham, Dorset, UK), BDH (Poole, Dorset, UK) or Fluka (Buchs, Switzerland).

Methods

Polyacrylamide gel electrophoresis. This was done on slab gels, 10–12% acrylamide, in Tris glycine buffer, pH 8.3, containing 0.1% sodium dodecyl sulphate [20]. Proteins were denatured in 3% sodium dodecyl sulphate, 0.1% dithiothreitol, 0.5 mM EDTA at 95°C for 2 min. Electrophoresis was for 30 min at 50 V, and then overnight at 100 V. Staining was with Coomassie brilliant blue R-250.

Enzyme assay. Glucose-6-phosphate dehydrogenase activity was determined spectrophotometrically at 340 nm, using a Beckman model Acta M-VI double-beam instrument with cells of 1-cm light path, maintained at 37°C. The reaction was started by adding enzyme to make a total volume of 3 ml containing 86 mM triethanolamine HCl buffer, pH 7.6, 6.7 mM $MgCl_2$, 1.2 mM glucose 6-phosphate, and 0.4 mM $NADP^+$. The reference cell contained the same mixture without enzyme.

Inactivation of the enzyme with acetylsalicylic acid. Baker's yeast glucose-6-phosphate dehydrogenase (43 μM with respect to subunit) was incubated with 6 mM acetylsalicylic acid in 70 mM Tris/HCl, pH 8.5, at 20°C. Samples from the reaction mixture were assayed for glucose-6-phosphate dehydrogenase activity and, when 16% of the original activity remained, ethanolamine/acetate pH 7.0 was added to a final concentration of 70 mM (to limit further reaction) and reagents were removed by dialysis against 50 mM sodium phosphate pH 7.0 and 0.1 mM dithiothreitol.

Samples for NMR spectroscopy. 10–12 mg/ml of enzyme were extensively dialysed against 50 mM potassium phosphate pH* 7.2 (meter reading uncorrected for the isotope effect on the glass electrode), 0.1 mM NaEDTA and 0.1 mM dithiothreitol in D_2O, and clarified by centrifugation. The concentration of $NADP^+$ used in the TRNOE experiments was 4.3 mM.

NMR spectroscopy. ^{1}H-NMR measurements were carried out at 270 MHz using a Bruker WH-270 spectrometer. 300 transients were averaged for each spectrum using 4096 data points for a 4.2 kHz spectral width; prior to Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz. The pulse sequence used for the TRNOE measurements was $(t_1 - \pi/2 - t_A - t_2)_n$ where the selective irradiation at a chosen frequency was applied during the time interval t_1 (0.002–0.8 s), t_A is the acquisition time (0.487 s) and t_2 is a delay (3 s) to allow for complete recovery of magnetization of all protons to their equilibrium values prior to perturbation by the selective radiofrequency field. The irradiation power used was sufficient to be in the high-power limit so that saturation can effectively be considered instantaneous, whilst preserving selectivity so that only a single averaged resonance at a time was saturated [19, 21]. It should also be noted that the band width of the applied radiofrequency field is $\approx 1/t$ (where t is the time for which it is applied), and the ratio of free to bound ligand was chosen so as to ensure that all initial slopes for the time development of the TRNOEs could be measured from irradiation times of $\gtrsim 50$ ms. Chemical shifts are expressed relative to 4,4-dimethylsilapentane-1-sulphonate. All measurements were carried out at 5°C.

RESULTS

The theoretical basis of the TRNOE has been extensively discussed previously [18, 19] and will therefore only be summarized here. In essence, the TRNOE involves the extension of NOE measurements [21–28] to exchanging systems such as ligand-protein complexes, making use of chemical exchange between the free and bound states of the ligand to transfer magnetic information concerning cross-relaxation between bound ligand protons from the bound state to the free state. In the case of free $NADP^+$ (i.e. in the absence of protein) no NOEs could be observed at irradiation times of less than 1 s. Thus, providing chemical exchange is fast on the chemical shift scale, the magnitude of the TRNOE, $N_{ij}(t)$, observed on the averaged ligand resonance i following irradiation of the averaged ligand resonance j for a short irradiation time t will be given by

$$N_{ij}(t) \approx -(1-a)\,\sigma_{ij}^{BB}\,t \qquad (1)$$

where σ_{ij}^{BB} is the cross-relaxation rate between the bound ligand protons and a the mole fraction of free ligand. If the bound ligand exists in more than one conformation, Eqn (1) is simply modified to

$$N_{ij}(t) \approx -\sum_x \lambda_x (\sigma_{ij}^{BB})_x\,t \qquad (2)$$

where λ_x is the mole fraction of bound ligand present in the xth conformation. Moreover, the sign of the TRNOE is negative as $\omega\tau_c \gg 1$ for the ligand-protein complex (where ω is the Larmor frequency and τ_c the correlation time). In contrast, in the case of the free ligand where $\omega\tau_c < 1$, the sign of the steady-state NOE is positive.

When dealing with large ligand-protein complexes, spin diffusion arising from highly effective indirect cross-relaxation between many protons [29] presents a potential problem. This can be overcome in one of two ways. The most effective approach simply involves measuring the initial slope of the time development of the TRNOE [19]. This, however, is very time-consuming. An alternative and quicker approach, therefore, consists of systematic pre-steady-state TRNOE measurements, irradiating at 10–20-Hz intervals throughout the region(s) of interest, using a constant short irradiation time, typically 0.4–0.5 s [17,30–33]. Under such conditions a plot of intensity of a particular resonance as a function of irradiation frequency yields an 'action spectrum' and apparent selectivity is maintained. This is because the extent of spin diffusion arising from indirect cross-relaxation via the protons of the protein is approximately independent of the irradiation frequency providing this is placed within the protein envelope [18, 19]. Nevertheless, small effects seen in such 'action spectra' should be treated with caution as they may still arise from indirect cross-relaxation and should therefore be checked by measuring the time dependence of the TRNOE.

Structural information on the conformation of the bound ligand is readily deduced from the TRNOE measurements as the cross-relaxation rate σ_{ij}^{BB} is proportional to $(r_{ij}^{BB})^{-6}$ where

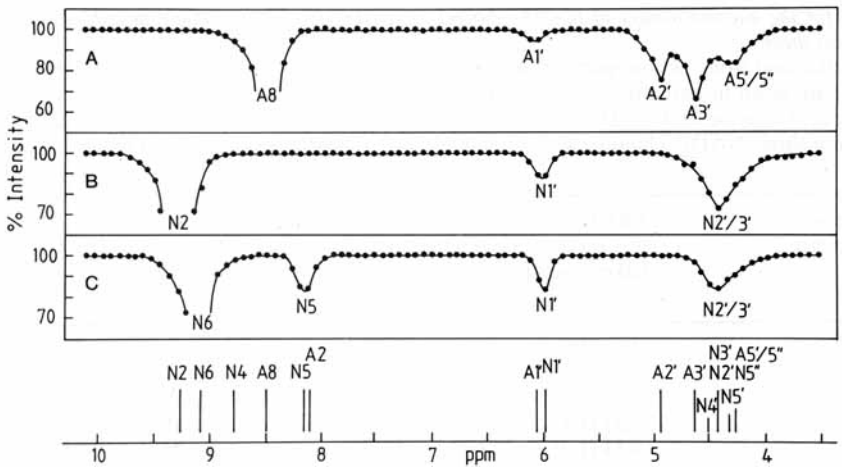

Fig. 1. *TRNOE 'action spectra' obtained by monitoring the intensity of the averaged H_A8 (A), H_N2 (B) and H_N6 (C) resonances of NADP$^+$ in the presence of bakers' yeast glucose-6-phosphate dehydrogenase as a function of irradiation frequency.* A stick diagram showing the positions of the averaged NADP$^+$ resonances is also shown. The TRNOE measurements were carried out using a 0.4-s pre-saturation pulse and by systematically irradiating the spectral region of 3.5 – 10 ppm at 20-Hz (≡ 0.074 ppm) intervals. The concentration of enzyme and NADP$^+$ employed was 12 mg/ml and 4.3 mM respectively. On the basis of a subunit M_r of 58000 and a single NADP$^+$ binding site per subunit, these conditions correspond to a ratio of free to bound NADP$^+$ of about 20. Temperature, 5 °C

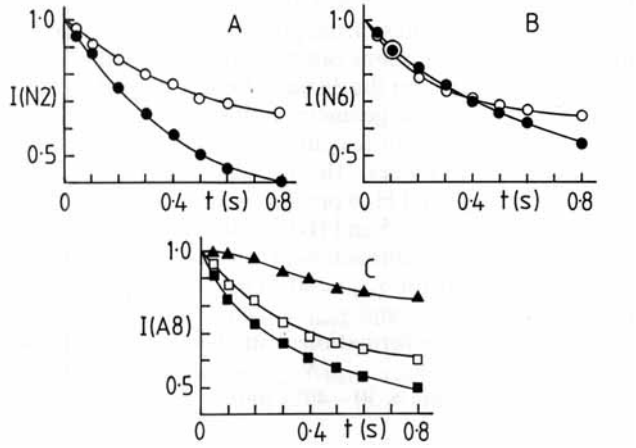

Fig. 2. *Time dependence of TRNOEs observed on the averaged H_N2 (A), H_N6 (B) and H_A8 (C) resonances of NADP$^+$ in the presence of bakers' yeast glucose-6-phosphate dehydrogenase following irradiation of the averaged H_N1' (○), $H_N2'/3'$ (●), H_A1' (▲), H_A2' (□) and H_A3' (■) ligand resonances.* The experimental conditions are the same as those in the Fig. 1 legend

r_{ij}^{BB} is the distance between the two bound ligand protons. Thus distance ratios can be obtained from the equation

$$r_{ij}^{BB}/r_{kl}^{BB} = \left(\sigma_{kl}^{BB}/\sigma_{ij}^{BB}\right)^{1/6} \approx [N_{kl}(t)/N_{ij}(t)]^{1/6} \qquad (3)$$

providing the correlation time of the two interproton vectors, $i-j$ and $k-l$, is the same. (Note that the approximation in Eqn (3) remains valid up to values of t 3 – 4-times longer than that in Eqns (1) and (2) [18, 19].)

Fig. 1 shows 'action spectra' for the H_A8, H_N2 and H_N6 resonances of NADP$^+$ (4.3 mM) in the presence of bakers' yeast glucose-6-phosphate dehydrogenase (≈ 12 mg/ml) obtained by systematically irradiating with a 0.4-s pre-saturation pulse throughout the spectral region of 3.5 – 10 ppm at 20 Hz (≡ 0.074 ppm) intervals. (Note that the subscripts A and N refer to the adenine and nicotinamide moieties of NADP$^+$ respectively.) In all cases the TRNOEs are maximal on irradiation at positions corresponding to free ligand resonances, indicating that chemical exchange between bound and free NADP$^+$ is fast on the chemical shift scale.

Considering the H_A8 resonance of the adenine ring first we observe negative TRNOEs of −33%, −23%, −13% and −5% at the positions of the averaged H_A3', H_A2', $H_A5'/5''$, and H_A1' ligand resonances respectively. Of these four TRNOEs, the first three arise from direct cross-relaxation whereas that between the H_A1' and the H_A8 protons arises as a result of indirect cross-relaxation. This is easily ascertained from the data in Fig. 2C which shows the time development of the TRNOEs observed on the averaged H_A8 ligand resonance following irradiation of the averaged H_A1', H_A2' and H_A3' ligand resonances. The time dependence of the TRNOEs between the H_A8 proton and the H_A2' and the H_A3' protons is characterised by an initial decrease in intensity of the averaged H_A8 ligand resonance indicative of a direct cross-relaxation process arising between protons separated by $\lesssim$ 0.4 nm [19]. In contrast, the time dependence of the TRNOE between the H_A8 and H_A1' protons exhibits a lag phase characteristic of an indirect cross-relaxation process arising between protons separated by $\gtrsim$ 0.4 nm [19]. Similar results are also obtained with bakers' yeast glucose-6-phosphate dehydrogenase inactivated with acetylsalicylic acid, and brewers' yeast and *Hansenula jadinii* glucose-6-phosphate dehydrogenases. The TRNOE data, together with the distance ratios calculated from them, for the adenine moiety of NADP$^+$ bound to these enzymes is summarized in Table 1. In all four cases, the data are indicative of an *anti* conformation about the glycosidic bond, with χ in the range 260 ± 20°, a sugar pucker conformation in the C3′-*endo* to O1′-*endo* range and a g^- or t conformation about the C4′-C5′ bond [31, 33]. This is illustrated in Fig. 3.

In the case of the nicotinamide moiety, the situation is somewhat more complex. Firstly, the averaged H_N2' and H_N3' resonances of the nicotinamide ribose are superimposed so that no information can be obtained on the sugar pucker conformation. Second, the magnitude of the direct TRNOEs (see Fig. 1 B, C and 2 A, B, and Table 2) observed between the

Table 1. *TRNOEs observed for the adenine moiety of NADP$^+$ bound to yeast glucose-6-phosphate dehydrogenase (G6PD), together with the distance ratios calculated from them*

The TRNOEs quoted were obtained from 'action spectra' using a presaturation time of 0.4 s. The initial slopes of the time dependence of the TRNOEs, where measured, are given in parentheses. The relative errors in the TRNOEs are $\lesssim \pm 0.15$. The error in the distance ratios is $\approx \pm 0.05$. The concentration of enzyme and NADP$^+$ employed was 9–12 mg/ml and 4.3 mM respectively. On the basis of a subunit of $M_r \approx 58000$ (see Fig. 4) and a single NADP$^+$ binding site per subunit, these conditions correspond to a ratio of free to bound ligand in the range $\approx 20-30$

Irradiated resonance	Observed resonance	TRNOE (initial slope of TRNOE) of G6PD from			
		bakers' yeast	brewers' yeast	*H. jadinii*	acetylsalicylic acid inactivated bakers' yeast
		% (s^{-1})			
H_A1'	H_A8	− 5 (lag)	− 3 (lag)	− 2 (lag)	0
H_A2'	H_A8	−23 (1.1)	−11 (0.7)	−18 (0.8)	− 5
H_A3'	H_A8	−33 (1.7)	−20 (0.9)	−26	−18
$H_A5'/5''$	H_A8	−13	−10	− 9	− 5
Distance ratios					
$r_{H_A3'-H_A8}/r_{H_A2'-H_A8}$		0.94	0.91	0.94	0.81
$r_{H_A5'/5''-H_A8}/r_{H_A2'-H_A8}$		1.0	1.02	1.12	1.00

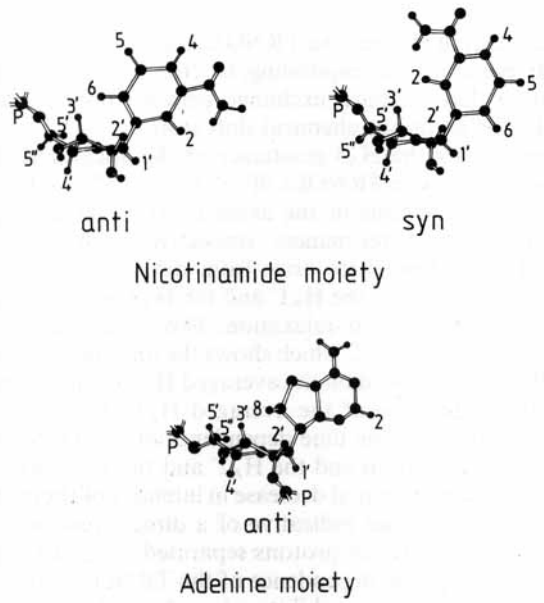

Fig. 3. *Conformations of the nicotinamide and adenine moieties of NADP$^+$ bound to yeast glucose-6-phosphate dehydrogenase binary complexes.* In the case of the nicotinamide moiety, the conformation about the glycosidic bond exists as a *syn/anti* mixture, whereas in the case of the adenine moiety only the *anti* conformer is found. The sugar pucker of the adenine ribose is 3′-*endo*. For simplicity, the sugar pucker of the nicotinamide ribose is also depicted as 3′-*endo*; however, no information on its sugar pucker could be obtained as the H_N2' and H_N3' resonances are superimposed

H_N2 protons and the H_N1' and H_N2'/H_N3' protons and between the H_N6 proton and the H_N1' and H_N2'/H_N3' protons for all four complexes is not compatible with a single conformation about the nicotinamide glycosidic bond. Instead, the data are indicative of a mixture of *syn* and *anti* conformations (see Fig. 3). By noting that the TRNOE ratios $N_{H_N1'-H_N2}/N_{H_N2'/3'-H_N6}$ and $N_{H_N1'-H_N6}/N_{H_N2'/3'-H_N6}$ are approximately equal, we conclude that the two glycosidic bond conformations differ by an about $\sim 180°$ ($\pm 20°$) flip of the nicotinamide ring. Approximate estimates of the glycosidic bond torsion angles for the two conformations can be deduced from the following three observations: (a) the distance between the H_N5 and H_N6 protons is fixed by the geometry of the nicotinamide ring and has a value of 0.25 nm (calculated on the basis of standard bond lengths and angles); (b) the magnitude of the TRNOE between the H_N1' and H_N6 protons is approximately the same as that between the H_N5 and H_N6 protons (see Table 2); (c) the distance of closest approach between the H_N1' and H_N6 protons is ≈ 0.23 nm for $\chi_{syn} = 60°$. On this basis, we conclude that $\chi_{syn} \approx 60 \pm 20°$ and $\chi_{anti} \approx 240 \pm 20°$. The proportion of *syn* and *anti* conformations can then be estimated from the TRNOE ratio $N_{H_N1'-H_N6}/N_{H_N1'-H_N2}$ which yields values of $\approx 60-70\%$ *syn* and $\approx 30-40\%$ *anti*.

DISCUSSION

Subunit size

Electrophoretic findings clearly establish (Fig. 4) that the glucose-6-phosphate dehydrogenases from the three yeasts all have subunits of roughly similar size with M_r in the region of 58000. Although this may not seen surprising, it is in fact at variance with reported values of 10000 [12], 14000 [12], and 49000 [34] for glucose-6-phosphate dehydrogenases from strains of *Hansenula jadinii*, with a value of 22000 [16] for the *Saccharomyces cerevisiae* enzyme, and with 51000 [35] for the enzyme from *Saccharomyces carlsbergensis* (an organism now regarded as a strain of *S. cerevisiae*, not a distinct species [17]). However, the value 61000 [34] for a *H. jadinii* glucose-6-phosphate dehydrogenase can be regarded as broadly in agreement with the present findings of around 58000, because values obtained by SDS/PAGE are always only an approximate guide to the true value (cf. [36]). Furthermore, values of 57000–65200 for the M_r of subunits from fungal [37] and mammalian [15, 38–41] sources, together with the present findings for yeasts, suggest a relatively uniform subunit M_r of round about 60000 for the glucose-6-phosphate dehydrogenases of eukaryotic organisms.

Table 2. *TRNOEs observed for the nicotinamide moiety of NADP$^+$ bound to yeast glucose-6-phosphate dehydrogenase (G6PD)*
The TRNOEs quoted were obtained from 'action spectra' using a presaturation time of 0.4 s. The initial slopes of the time dependence of the TRNOEs, where measured, are given in parentheses. The relative error in the TRNOEs and initial slopes is $\lesssim \pm 0.15$. The relative error in the ratios of TRNOEs and initial slopes is $\lesssim \pm 0.3$. The experimental conditions are the same as those in Table 1, namely a ratio of free to bound ligand in the range $\approx 20-30$

Irradiated resonance	Observed resonance	TRNOE (initial slope of TRNOE) of G6PD from			
		Bakers' yeast	Brewers' yeast	*H. jadinii*	acetylsalicylic acid inactivated bakers' yeast
		% (s^{-1})			
H_N1'	H_N2	−12 (0.9)	− 7 (0.5)	−11 (0.6)	− 7 (0.7)
H_N1'	H_N6	−16 (1.4)	−13 (0.7)	−20 (1.5)	− 9 (1.0)
H_N2'/H_N3'	H_N2	−27 (1.6)	−18 (1.0)	−24 (1.1)	−11 (1.2)
H_N2'/H_N3'	H_N6	−15 (1.2)	− 7 (0.6)	−13 (0.6)	−13 (0.6)
H_N5	H_N6	−15 (1.0)	− 9 (0.8)	−15 (0.7)	(0.6)
TRNOE ratios (ratios of initial slopes)					
$N_{H_N1'-H_N2}/N_{H_N2'/3'-H_N6}$		0.8 (0.8)	1.0 (0.8)	0.9 (1.0)	0.9 (1.4)
$N_{H_N1'-H_N6}/N_{H_N2'/3'-H_N2}$		0.6 (0.9)	0.7 (0.7)	0.8 (1.4)	0.8 (0.8)
$N_{H_N1'-H_N6}/N_{H_N1'-H_N2}$		1.3 (1.6)	1.9 (1.4)	1.8 (2.5)	1.3 (1.4)

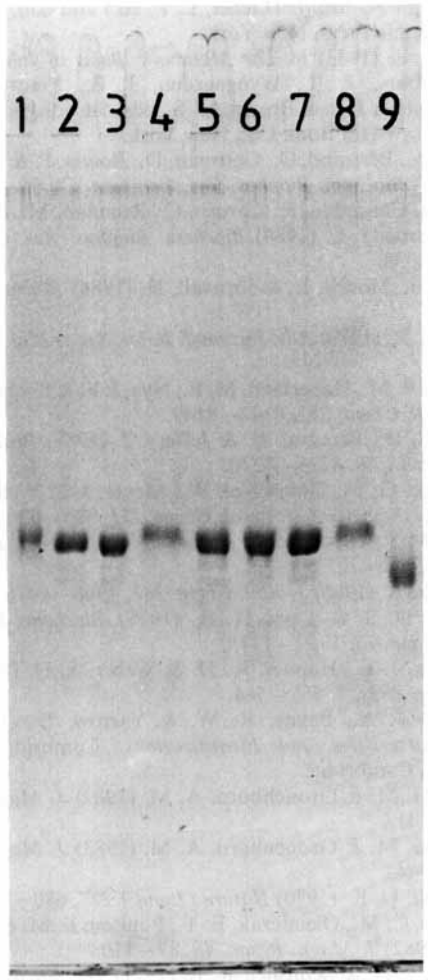

Fig. 4. *Sodium dodecylsulphate/polyacrylamide gel electrophoresis of glucose-6-phosphate dehydrogenases from three yeasts showing that the subunit* M_r *are about 58000.* Gel lanes: (1) *H. jadinii*, (2) brewers yeast, (3) bakers' yeast, (4) bovine catalase (M_r 58100), (5) *H. jadinii* plus brewers' yeast, (6) *H. jadinii* plus bakers' yeast, (7) brewers' yeast plus bakers' yeast, (8) bovine catalase, (9) bovine glutamate dehydrogenase (M_r 55393). Gel, 12% polyacrylamide. Stain, Coomassie blue. Top, cathode; bottom, anode

Coenzyme binding and structure-function relationships

From the TRNOE measurements it is seen that the conformation of NADP$^+$ binding is similar for all three enzymes, suggesting functional similarities. Our results show that in enzyme-NADP$^+$ binary complexes both *syn* and *anti* conformations about the nicotinamide glycosidic bond of the bound coenzyme are present (Fig. 3). This might be of significance with respect to the catalytic function. When reaction occurs in the catalytically competent complex of the *S. cerevisiae* enzyme, hydrogen is transferred from C-6 of β-D-glucopyranose 6-phosphate [42] to the 4-*pro-S* position of NADP$^+$ [43]. (The reaction shows the same specificity also for the enzymes from other organisms investigated [44–49].) The alignment that results in this stereospecificity could in principle (but see below) involve either the *syn* or the *anti* conformation, but not both conformations, so a critical change restricting the coenzyme to one of these conformations occurs after the binary complex stage. If hydrogen transfer is the rate-limiting step [50], then functionally necessary conformational changes must occur rapidly within a ternary complex, or during ternary complex formation.

Interestingly, a small proportion of *anti* conformation was detected in the population of predominantly *syn* binary complexes for NADP$^+$ and especially NAD$^+$ with *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase [51], though various 4-*pro-R*-specific enzymes showed only the *anti* conformation in binary complexes with NADP$^+$ (dihydrofolate reductase [52]) and NAD$^+$ (alcohol dehydrogenases [31], lactate dehydrogenase [51], and, notwithstanding other conformational differences, also sorbitol dehydrogenase [53]).

Evolutionary considerations led to a view that 4-*pro-R*-specific dehydrogenases should generally use coenzyme bound in the *anti* conformation, whereas the conformation should be *syn* in dehydrogenases with 4-*pro-S* specificity [54]. The suggestion that for optimal catalysis, chemically less-reactive carbonyls require the more reactive *syn* conformation of coenzyme [55] implied a *syn* or *anti* arrangement influenced by the reaction catalysed, and, taken together with the correlation mentioned above [54], implied that whether the steric course is 4-*pro-R* or 4-*pro-S* depends upon the reaction

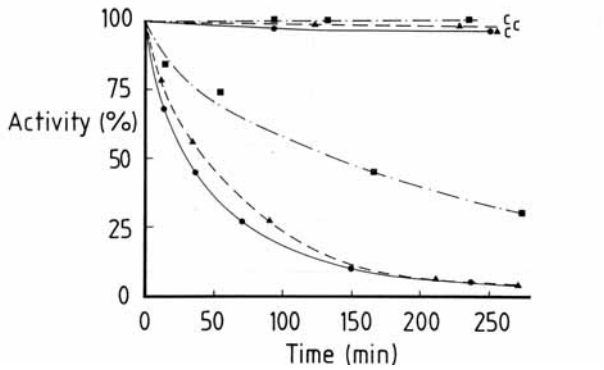

Fig. 5. *Effect of substrate and of coenzyme on the inactivation of bakers' yeast glucose-6-phosphate dehydrogenase by acetylsalicylic acid.* Incubation of enzyme (135 nM with respect to subunit) with acetylsalicylic acid (5.8 mM) in 70 mM Tris/HCl, 100 μM dithiothreitol, pH 8.5, 20°C, caused slow inactivation (●——●). The initial inactivation rate was only slightly slower in the presence of $NADP^+$ (360 μM) (▲– – –▲), whereas glucose 6-phosphate (360 μM) (■—·—■) had a more marked effect. In the absence of acetylsalicylic acid (curves marked c), there was little loss of activity (●——●), whether or not $NADP^+$ (▲– – –▲) or glucose 6-phosphate (■—·—■) was present

catalysed. According to this argument, all glucose-6-phosphate dehydrogenases can be expected to bind coenzyme in the *syn* conformation and to transfer hydrogen with 4-*pro-S* specificity. The present findings establish that evidence for the conformations in relevant ternary complexes is essential to substantiate these points.

Inactivation of bakers' yeast glucose-6-phosphate dehydrogenase with acetylsalicylic acid

Effect of inactivation on the conformation of bound $NADP^+$. Bakers' yeast glucose-6-phosphate dehydrogenase that had been inactivated by reaction with acetylsalicylic acid formed a binary complex in which the conformation of bound $NADP^+$ differed little, if at all, from those in the complexes with the active enzyme (see Table 2). The inactivation is accompanied by acetylation of the ε-amino group of a particular lysine residue [8], and the present findings therefore show that this ε-amino group does not have an essential role in binary complex formation with $NADP^+$.

Effect of substrate and of coenzyme on the inactivation reaction. Inactivation of bakers' yeast glucose-6-phosphate dehydrogenase by acetylsalicylic acid proceeded marginally more slowly in the presence of 360 μM $NADP^+$ (Fig. 5). On the other hand, 360 μM glucose-6-phosphate clearly lowered the inactivation rate. The K_m values reported for $NADP^+$ (about 5 μM [9]) and for glucose 6-phosphate (about 30 μM [9]), as well as directly determined thermodynamic dissociation constants [56], are considerably lower than the concentrations used in the present protection experiments (roughly 70-fold, and 12-fold, respectively). The only small effect of $NADP^+$ on inactivation therefore suggests that the $NADP^+$-glucose-6-phosphate-dehydrogenase binary complex may undergo acetylation in a similar way to the free enzyme, consistent with the finding (Table 2) of a substantially unaltered $NADP^+$ conformation in the binary complex of $NADP^+$ with the acetylsalicylic-acid-inactivated enzyme. All evidence therefore indicates that the lysine residue involved in the inactivation [8] is not located at the coenzyme binding site. The small but distinct protective effect of glucose 6-phosphate shows that this lysine residue is in a part of the molecule affected by glucose-6-phosphate binding. This need not be the glucose-6-phosphate binding region itself. Evidence suggests that the glucose-6-phosphate dehydrogenase reaction in this case follows a random course [56]. It is quite likely that a change in the conformation of the enzyme occurs upon formation of a glucose-6-phosphate–enzyme binary complex, and altered reactivity towards acetylsalicylic acid could then result from this conformational change.

We thank the Medical Research Council (GMC and AMG), the Lister Institute for Preventive Medicine (GMC), the University of Aberdeen (JJ and LH), and the Wellcome Trust (JJ and LH) for financial support. GMC is a Lister Institute Research Fellow.

REFERENCES

1. Yoshida, A. & Beutler, E. (1983) *Ann. Hum. Genet. 47*, 25–38.
2. Luzzatto, L. (1979) *Blood 54*, 961–976.
3. Beutler, E. (1978) in *Hemolytic Anemia in Disorders of Red Cell Metabolism*, Plenum Press, New York.
4. Mager, J., Chevion, M. & Glaser, G. (1980) in *Toxic Constituents of Plant Foodstuffs* (Liener, L. I., ed.) 2nd edn, pp. 265–294, Academic Press, New York.
5. Beutler, E. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Fredrikson, D. S., Goldstein, J. L. & Brown, M. S., eds) 5th edn, pp. 1629–1653, McGraw-Hill Book Co., New York.
6. Kahn, A., Bertrand, O., Cottreau, D., Boivin, P. & Dreyfus, J.-C. (1977) *Biochem. Biophys. Res. Commun. 77*, 65–72.
7. Descalzi-Cancedda, F., Caruso, C., Romano, M., di Prisco, G & Camardella, L. (1984) *Biochem. Biophys. Res. Commun. 118*, 332–338.
8. Jeffery, J., Hobbs, L. & Jörnvall, H. (1984) *Biochemistry*, in the press.
9. Levy, H. R. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 48*, 97–192.
10. Austen, B. M., Haberland, M. E., Nye, J. F. & Smith, E. L. (1977) *J. Biol. Chem. 252*, 8142–8149.
11. Jörnvall, H., Persson, M. & Jeffery, J. (1981) *Proc. Natl Acad. Sci. USA 78*, 4226–4230.
12. Domagk, G. F., Domschke, W., Meyer, J. & Weise, M. (1970) *Hoppe-Seyler's Z. Physiol. Chem. 351*, 923–926.
13. Kanji, M. I., Toews, M. L. & Carper, W. R. (1976) *J. Biol. Chem. 251*, 2255–2257.
14. Yoshida, A. (1966) *J. Biol. Chem. 241*, 4966–4976.
15. Shreve, D. S. & Levy, H. R. (1977) *Biochem. Biophys. Res. Commun. 78*, 1369–1375.
16. Robbins, J. E., Hapner, K. D. & Weber, S. D. (1975) *Physiol. Chem. Phys. 7*, 555–564.
17. Barnett, J. A., Payne, R. W. & Yarrow, D. (1983) *Yeasts: Characteristics and Identifications*, Cambridge University Press, Cambridge.
18. Clore, G. M. & Gronenborn, A. M. (1982) *J. Magn. Reson. 48*, 402–417.
19. Clore, G. M. & Gronenborn, A. M. (1983) *J. Magn. Reson. 53*, 423–442.
20. Laemmli, U. K. (1970) *Nature (Lond.) 277*, 680–685.
21. Dobson, C. M., Olejniczak, E. T., Poulsen, F. M. & Ratcliffe, R. G. (1982) *J. Magn. Reson. 48*, 87–110.
22. Noggle, J. H. & Schirmer, R. E. (1971) *The Nuclear Overhauser Effect – Chemical Applications*, Academic Press, New York.
23. Redfield, A. G. & Gupta, K. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 405–419.
24. Poulsen, F. M., Hosch, J. C. & Dobson, C. M. (1980) *Biochemistry 19*, 2597–2607.
25. Wagner, G., Kumar, A. & Wüthrich, K. (1981) *Eur. J. Biochem. 115*, 375–384.
26. Roy, S. & Redfield, A. G. (1983) *Biochemistry 22*, 1386–1390.

27. Clore, G. M. & Gronenborn, A. M. (1983) *EMBO J. 2*, 2109–2115.
28. Braun, W., Wider, G., Lee, K. H. & Wüthrich, K. (1983) *J. Mol. Biol. 169*, 921–948.
29. Kalk, A. & Berendsen, H. C. J. (1976) *J. Magn. Reson. 24*, 343–366.
30. Gronenborn, A. M. & Clore, G. M. (1982) *Biochemistry 21*, 4040–4048.
31. Gronenborn, A. M. & Clore, G. M. (1982) *J. Mol. Biol. 157*, 155–160.
32. Clore, G. M. & Gronenborn, A. M. (1982) *FEBS Lett. 145*, 197–202.
33. Clore, G. M., Gronenborn, A. M., Mitchison, C. & Green, N. M. (1982) *Eur. J. Biochem. 128*, 113–117.
34. Chilla, R. & Domagk, G. F. (1973) *Arch. Int. Physiol. Biochim. 81*, 583.
35. Yue, R. H., Noltmann, E. A. & Kuby, S. A. (1969) *J. Biol. Chem. 244*, 1353–1364.
36. de Jong, W. W., Zweers, A. & Cohen, L. H. (1978) *Biochem. Biophys. Res. Commun. 82*, 532–539.
37. Scott, W. A. (1971) *J. Biol. Chem. 246*, 6353–6359.
38. Hizi, A. & Yagil, G. (1974) *Eur. J. Biochem. 45*, 201–209.
39. Nevaldine, B. H., Hyde, C. M. & Levy, H. R. (1974) *Arch. Biochim. Biophys. 165*, 398–406.
40. Singh, D. & Squire, P. G. (1974) *Biochemistry 13*, 1819–1825.
41. Donohue, T. M., Jr, Mahowald, T. A., Adams, D. J. & Baker, K. L. (1981) *Biochim. Biophys. Acta 658*, 356–368.
42. Salas, M., Vinuela, E. & Sols, A. (1965) *J. Biol. Chem. 240*, 561–568.
43. Stern, B. K. & Vennesland, B. (1960) *J. Biol. Chem. 235*, 205–208.
44. Lessmann, D., Schmiz, K.-L. & Kurz, G. (1975) *Eur. J. Biochem. 59*, 545–559.
45. Smith, J. E. & Beutler, E. (1966) *Proc. Soc. Exp. Biol. Med. 122*, 671–673.
46. Malaisse, W. J., Sener, A., Koser, M. & Herchuelz, A. (1976) *J. Biol. Chem. 251*, 5936–5943.
47. Mazover, A. & Benizman, M. (1973) *FEBS Lett. 36*, 203–206.
48. Davies, D. D., Teixeira, A. & Kenworthy, P. (1972) *Biochem. J. 127*, 335–343.
49. Arnold, L. J., You, K., Allison, W. S. & Kaplan, N. O. (1976) *Biochemistry 15*, 4844–4849.
50. Rose, I. A. (1961) *J. Biol. Chem. 236*, 603–609.
51. Levy, H. R., Ejchart, A. & Levy, G. C. (1983) *Biochemistry 22*, 2792–2796.
52. Feeney, J., Birdsall, B., Roberts, G. C. K. & Burgen, A. S. V. (1983) *Biochemistry 22*, 628–633.
53. Gronenborn, A. M., Clore, G. M. & Jeffery, J. (1984) *J. Mol. Biol. 172*, 559–572.
54. You, K., Arnold, L. J., Allison, W. S. & Kaplan, N. O. (1978) *Trends Biochem. Sci. 3*, 265–268.
55. Benner, S. A. (1982) *Experientia (Basel) 38*, 633–637.
56. Kuby, S. A., Wu, J. T. & Roy, R. N. (1974) *Arch. Biochem. Biophys. 165*, 153–178.

A. M. Gronenborn and G. M. Clore, Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, D-8033 Martinsried, Federal Republic of Germany

L. Hobbs and J. Jeffery, Department of Biochemistry, University of Aberdeen, Marischal College, Aberdeen, Grampian, Scotland AB9 1AS